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Targeting innate immunity during the neonatal period with NOD2-based ligands to fight Cryptosporidiosis

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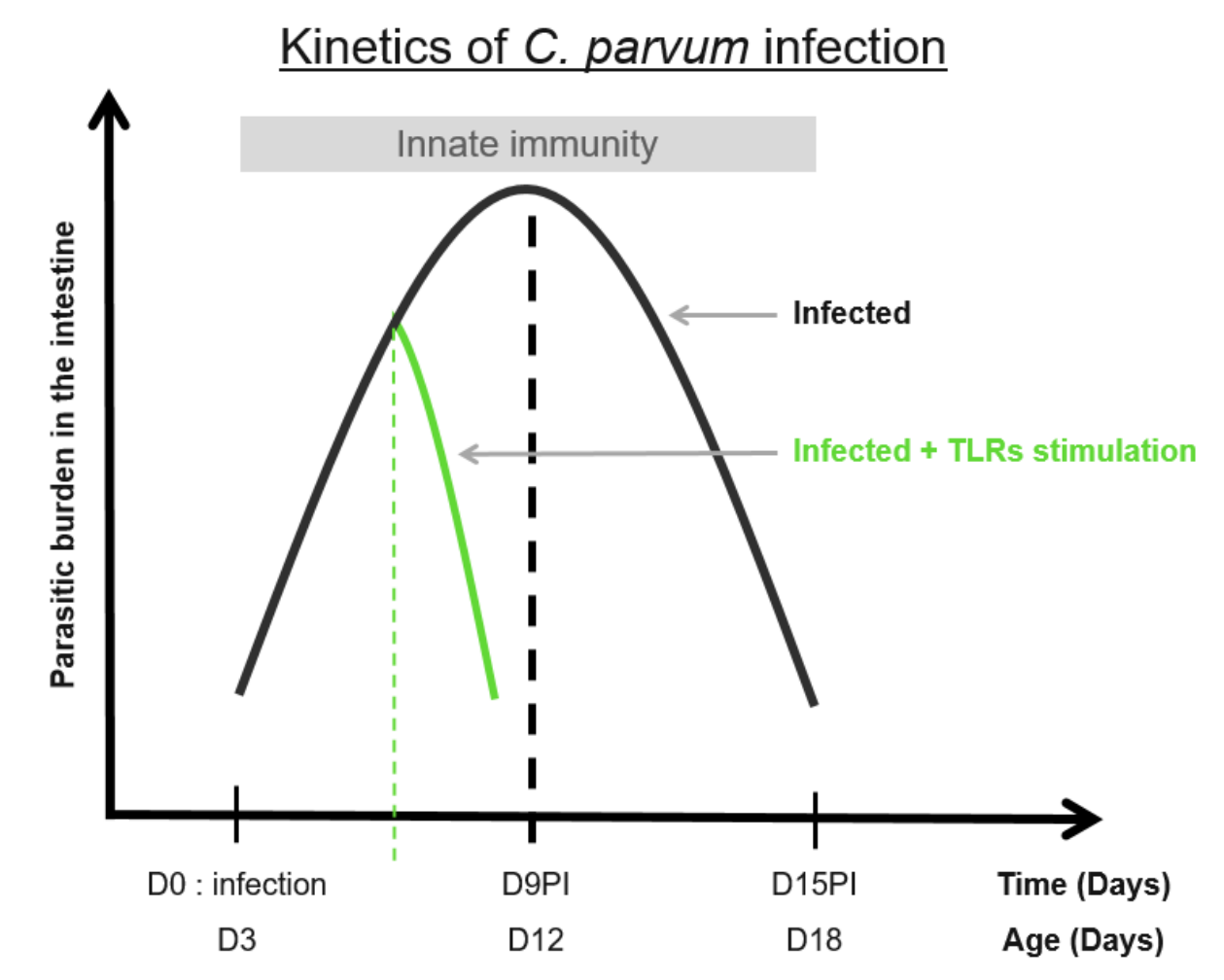
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Context

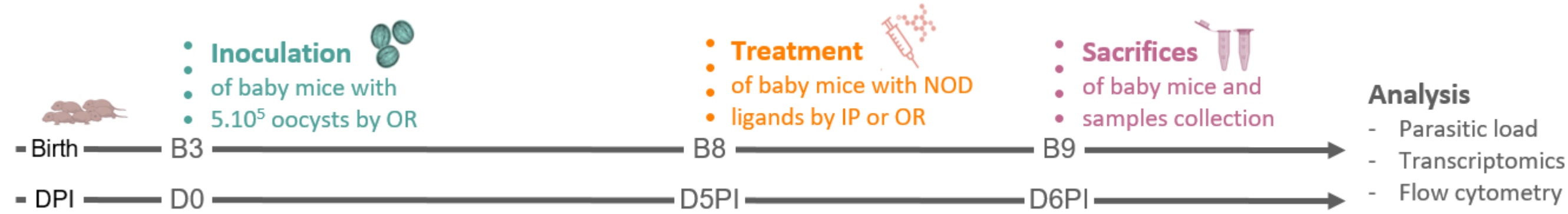
During the **neonatal period**, the immune system is still immature, giving at newborns susceptibility to infections such as Cryptosporidiosis, caused by the parasite *Cryptosporidium parvum* (*C. parvum*). This zoonosis is due to an orally transmitted protozoan that develops in intestinal epithelial cells and causes acute diarrhea in very young ruminants. Cryptosporidiosis also causes a moderate-to-severe diarrhea in children under 5 years and immune-compromised hosts. **Today, there is no vaccine to prevent infection nor efficient treatment, and there is therefore an urgent need for the development of new methods to control this zoonosis.**



The innate immunity has been shown to play an important role in the control of *C. parvum* infection (Lacroix-Lamandé & Laurent, 2017). **We hypothesize that modulation of the innate immune system during the neonatal period could enhance the immune defense at young ages** and favor the control of cryptosporidiosis. We have previously shown that the immunostimulation with ligands of the innate immunity targeting the Toll Like Receptors (TLRs) can help the mice to control *C. parvum* infection. (Barrier et al., 2006 ; Lacroix-Lamandé et al. 2009 ; Lantier et al., 2014 ; Lacroix-Lamandé et al., 2014).

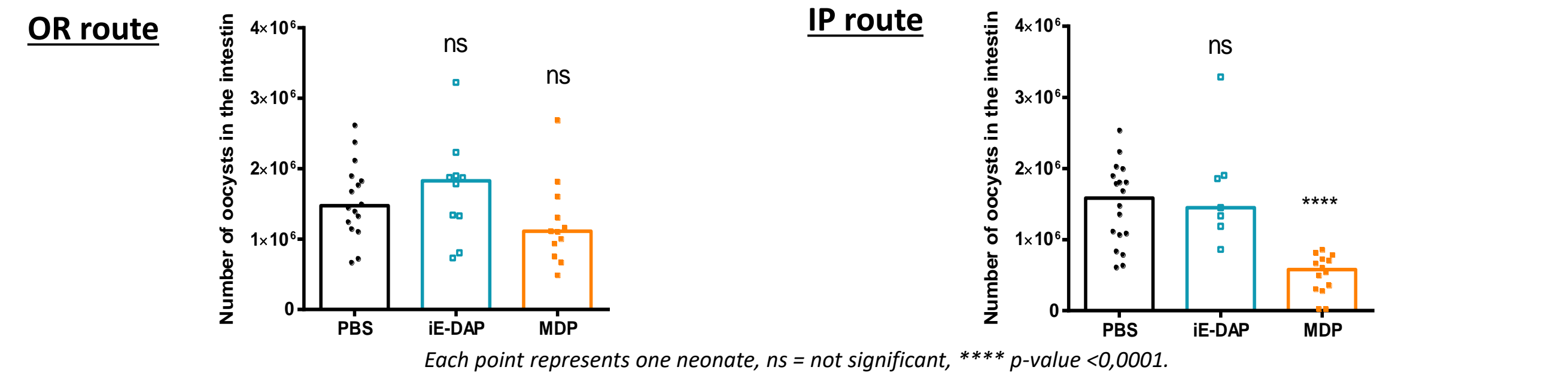
Nucleotides Oligomerization Domain (**NODs**) are other innate immune receptors expressed by diverse immune cells (monocytes, dendritic cells, eosinophils, and neutrophils) and by epithelial cells. They recognize peptidoglycan of bacteria. In the intestine, these receptors are present on the different types of intestinal epithelial cells (enterocytes, Paneth cells and Goblet cells) and are essential in the maintenance of intestinal homeostasis. **In this work, we studied if, during the neonatal period, the stimulation of these NOD receptors could efficiently stimulate the innate immune responses and confer protection against an enteric infection by *C. parvum*.**

Experimental Design



Neonatal mice are infected at 3 day-old with 5.10^5 oocysts of *C. parvum* by oral route (OR). They receive iE-DAP (di-aminopimelic acid containing peptides), a NOD1 agonist or MDP (muramyl-dipeptide), a NOD2 agonist by intraperitoneal (IP) or oral (OR) route. 24 hours post treatment, mice were sacrificed and samples were collected.

A – Protection against *C. parvum*

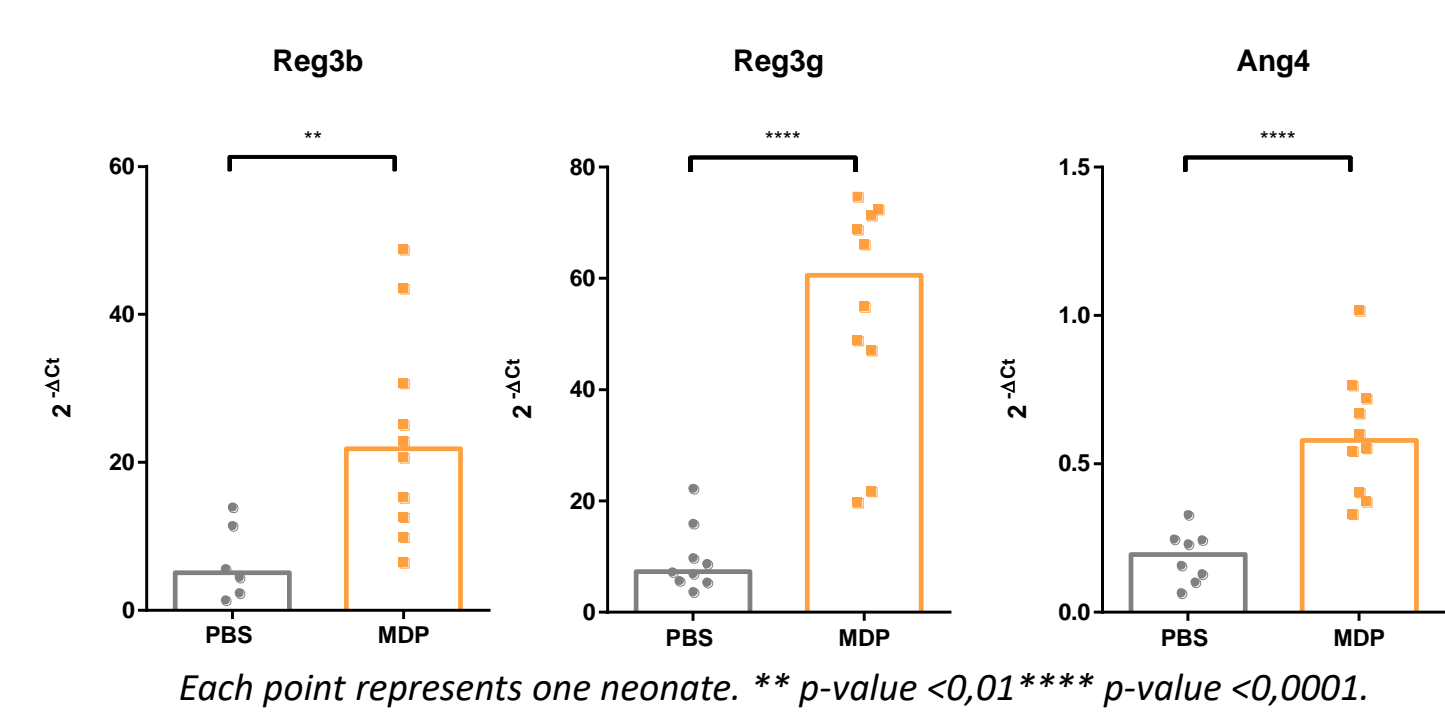


The MDP (NOD2) treatment significantly reduced the parasitic burden in the intestines of neonatal mice when administered by IP route. iE-DAP (NOD1) has no effect.

B – Transcriptomic response in the intestine of neonatal mice

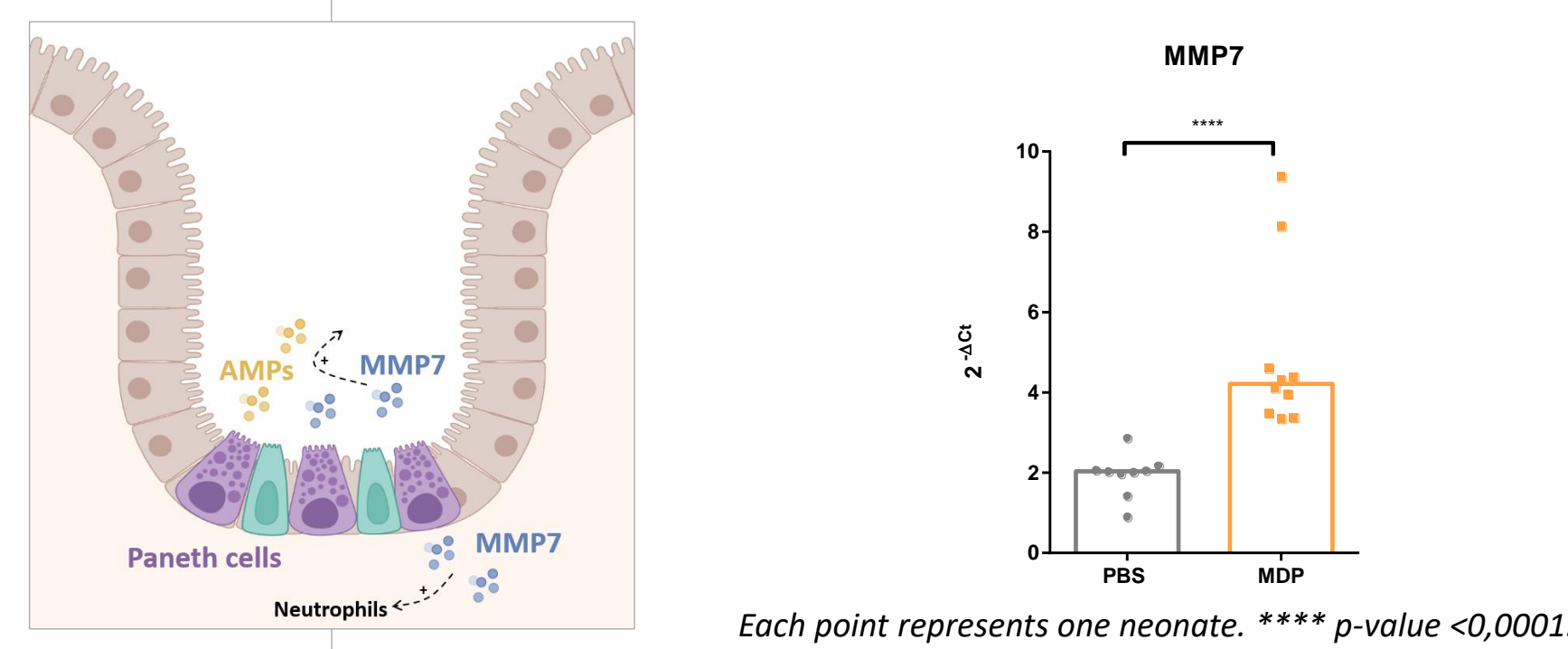
To decipher the mechanism of protection induced by MDP treatment by IP route, transcriptomics studies were performed in intestinal tissue. Among the 96 genes studied, the genes that were significantly regulated are described below.

Antimicrobial peptides - AMPs



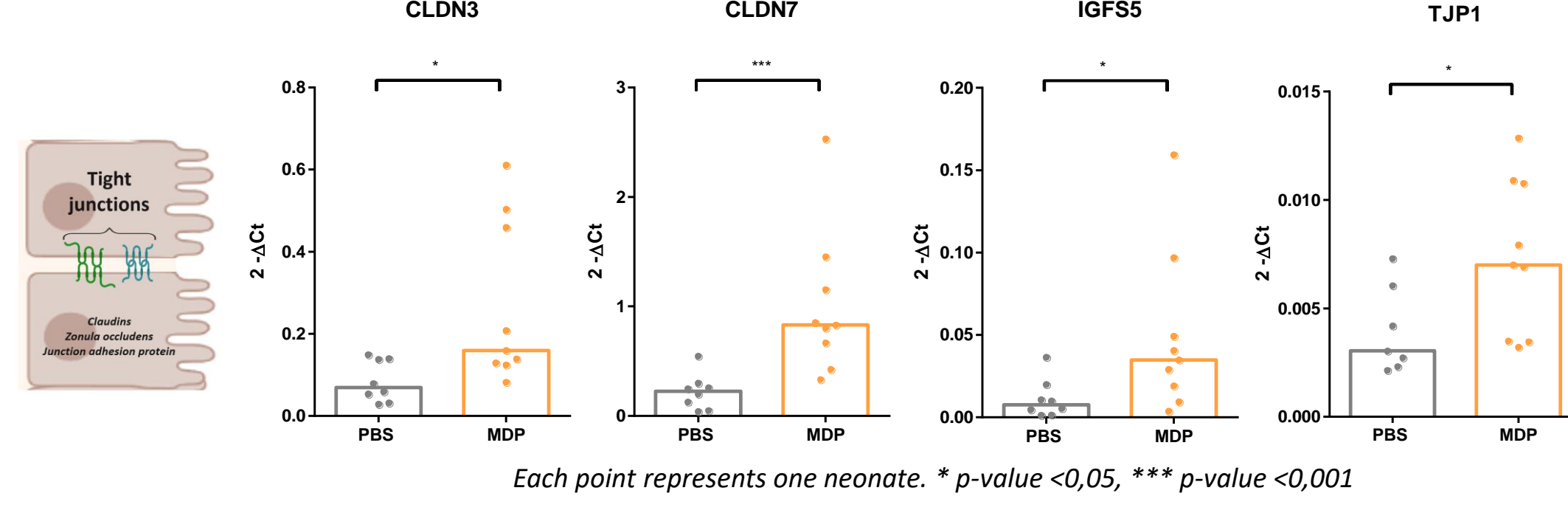
Gene expression of Regenerating islet-derived protein III (**Reg3**) β and γ , and Angiogenin 4 (**Ang4**) are significantly increased in the ileum of mice after MDP treatment. These AMPs are mainly produced by the Paneth cells (intestinal epithelial cells located in the intestinal crypt).

Matrix metalloproteinase-7 – MMP7



MMP7 gene expression is significantly increased in the ileum of MDP treated mice. MMP7 is a member of the matrix metalloproteinase family, this enzyme is produced by the Paneth cells and is responsible for the cleavage of α -defensins (AMPs) and was shown to induce neutrophils influx into tissues.

Tight junctions

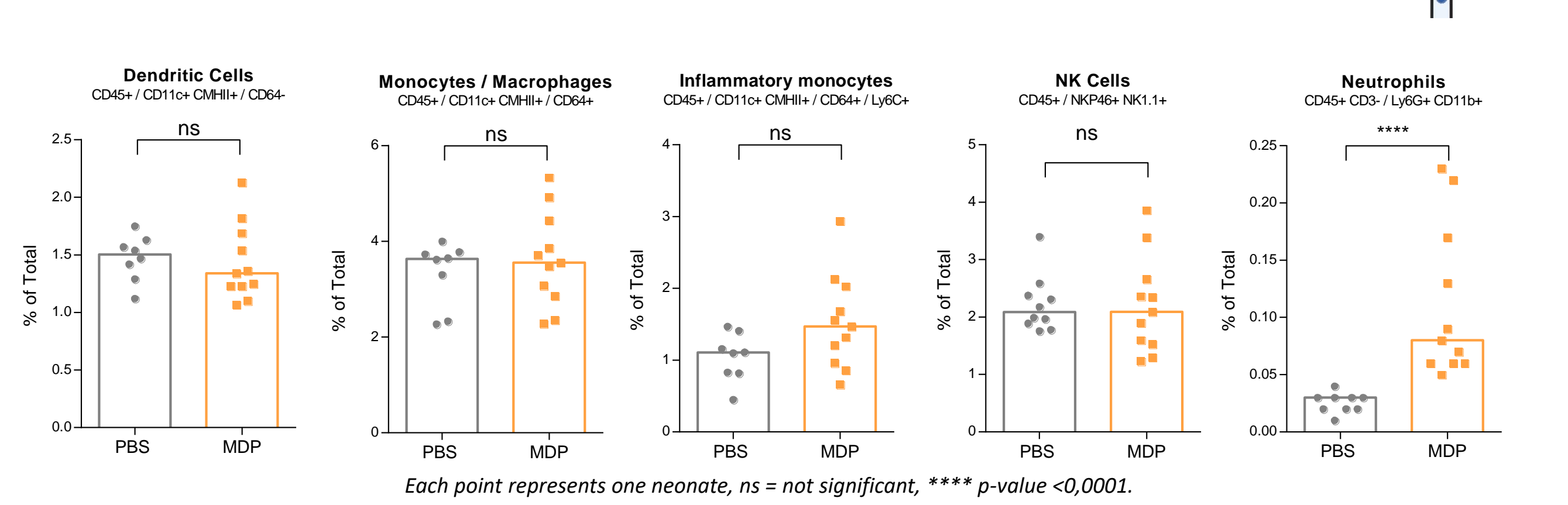


Gene expression of **tight junction** proteins is significantly increased following MDP treatment in the ileum of neonatal mice. *CLDN3*, claudine 3; *CLDN7*, claudine 7; *IGFS4* = *JCAM* = *Jam4*, junctional adhesion molecule 4; *TJP1*, tight junction protein 1

Inflammatory cytokines No modulation of the gene expression for the inflammatory cytokines in the ileum of the mice 24h post MDP treatment was observed (IFN γ , TNF α , IL-6, IL-1 β , IL-12).

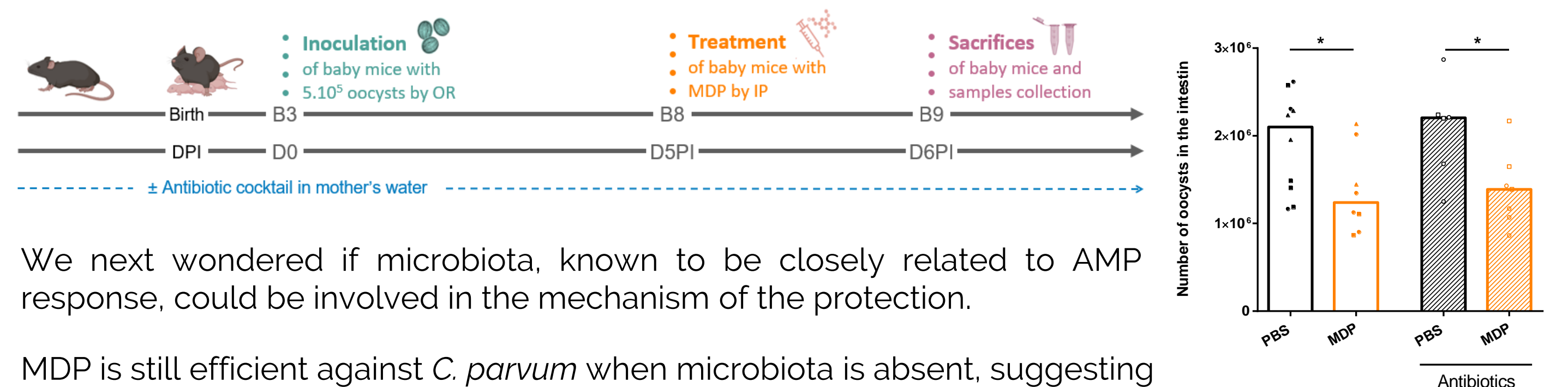
C – Immune cell recruitment in the intestine of neonatal mice

The recruitment of immune cells in the lamina propria was analyzed after tissue dissociation, cell isolation and immunofluorescent stainings by specific combinations of antibodies. These cells were then analyzed by flow cytometry.



Among the immune cells studied in the lamina propria, only **neutrophils** (CD45+ CD11b+ Ly6G+) are increased in the lamina propria of the infected neonatal mice that received MDP treatment.

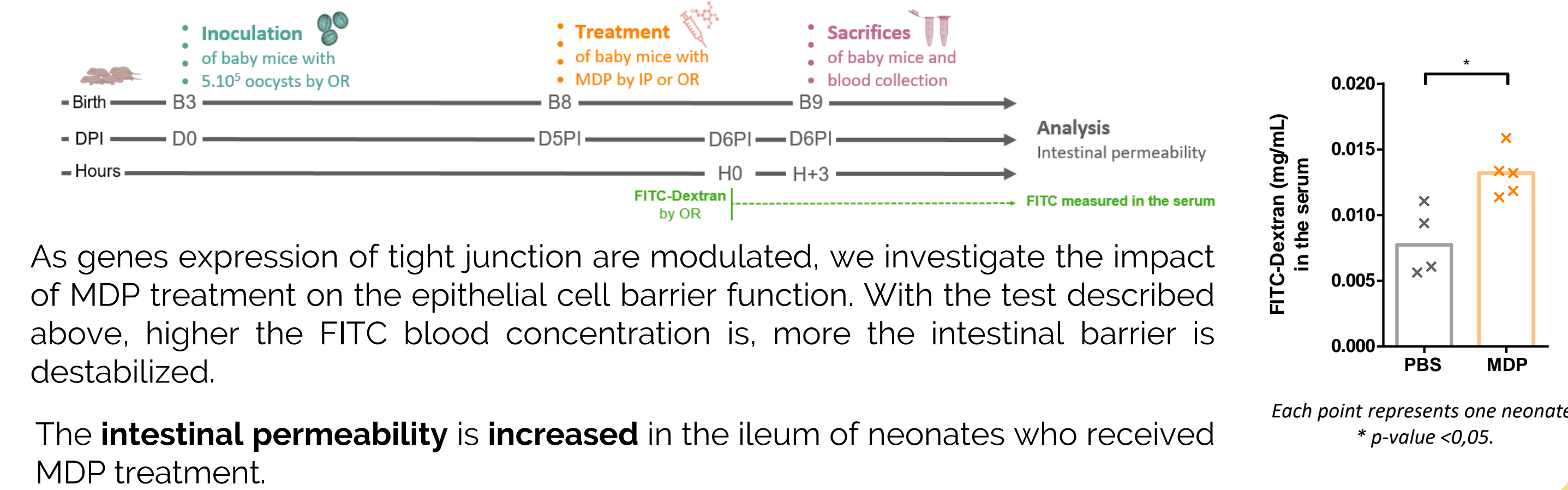
D – Role of intestinal microbiota on the protection



We next wondered if microbiota, known to be closely related to AMP response, could be involved in the mechanism of the protection.

MDP is still efficient against *C. parvum* when microbiota is absent, suggesting that the protection against *C. parvum* induced by MDP **do not require microbiota**.

E- Impact of MDP stimulation on the intestinal epithelial barrier



As genes expression of tight junction are modulated, we investigate the impact of MDP treatment on the epithelial cell barrier function. With the test described above, higher the FITC blood concentration is, more the intestinal barrier is destabilized.

The intestinal permeability is increased in the ileum of neonates who received MDP treatment.

Conclusions

The stimulation of the NOD2 receptor during the neonatal period induces an intestinal protective response against the parasite *C. parvum*. If the deciphering of the precise protective mechanism still needs complementary experiments, different hypothesis can already be made.

AMPs and **Paneth cells** are known to play a role in the control of *C. parvum* (Guesdon et al., 2016 ; non published data). Our data suggest that stimulation of these cells with NOD2 ligand can favor AMP and MMP7 response by Paneth cells and therefore participate in the protection. Moreover, the increased response of **MMP7** could be associated to the **neutrophils recruitment** and consequently to the **increased permeability** detected after MDP stimulation. **All together these observations suggest a reepithelization process that could allow parasite elimination.**